



Use of selected bacteria and yeast to protect gnotobiotic *Artemia* against different pathogens

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Abstract

To evaluate the potential probiotic effect of two bacterial strains towards *Artemia* cultured in different gnotobiotic conditions, challenge tests were performed with a virulent *Vibrio campbellii* or with an opportunistic *Vibrio proteolyticus* strain. For that purpose, three feed sources (different isogenic *Saccharomyces cerevisiae* mutant strains) were chosen, yielding distinct *Artemia* culture performances. Both bacterial strains, selected from previous well-performing *Artemia* cultures, were able to protect against the opportunistic *V. proteolyticus*, while, generally, these bacteria could not protect *Artemia* against *V. campbellii*. The quality of the feed provided (in the form of the isogenic mnn9 yeast mutant) to *Artemia* had a stronger influence on nauplii protection against the opportunistic and the virulent *Vibrio* than the addition of beneficial bacteria. This feed has a higher nutritional value for *Artemia*, but contains also more cell wall bound β -glucans and chitin. Data suggest that the change in the cell wall composition, rather than the overall better nutritional value, of the mnn9 strain is responsible for the protection against both *Vibrios*.

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1. Introduction

Live feeds, such as *Artemia*, play an important role in the dietary regime of fish and shellfish larvae produced in industrial hatcheries (Sorgeloos et al., 1986). However, these feeds can be a source of pathogenic bacteria in the hatchery environments (Vaseeharan and Ramasamy, 2003); hence, prevention of disease spreading through the feed animal is essential in aquaculture.

Several environmental-friendly prophylactic and preventive methods can putatively be used to control pathogenic bacteria and to maintain a healthy microbial environment in aquaculture systems (e.g. probiotics, immunostimulants, antimicrobial peptides, and quorum sensing systems: Sakai, 1999; Verschuere et al., 2000; Bachère, 2003; Defoirdt et al., 2005). However, applications of these technologies must be based on thorough understanding of mechanisms involved and the putative consequences. An essential part of that understanding can be provided by looking in detail at host–microbial interactions.

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A key experimental strategy to study these interactions is to first define the functioning of the host in the absence of bacteria and then to evaluate the effects of adding a single or defined population of microbes, or certain compounds (i.e., under gnotobiotic conditions) (Gordon and Pesti, 1971). *Artemia* is particularly useful as a test organism to study the host–microbe interactions (e.g. to evaluate potential probiotic bacteria before testing in target-organisms), as it can easily be cultured in gnotobiotic conditions (Marques et al., 2004a,b). Furthermore, it is possible to bioencapsulate *Artemia* with probiotic bacteria in different gnotobiotic environments (e.g. Rico-Mora and Voltolina, 1995; Verschuere et al., 1999, 2000; Orozco-Medina et al., 2002). Nonetheless, most studies reported in literature have been performed with *Artemia* cultured in poor conditions, such as using only bacteria as feed (Rico Mora and Voltolina, 1995; Makridis et al., 2000), or using autoclaved (Douillet, 1987; Orozco-Medina et al., 2002) or irradiated inert feed (Verschuere et al., 1999, 2000). Such artificial environments in combination with poor feed negatively influence the overall condition of gnotobiotically grown *Artemia*. According to Marques et al. (2005), when medium/good-quality feeds are provided to *Artemia*, both direct (probiotic or pathogenic) and indirect (nutritional) effects of a bacterial strain are less visible than with poor-quality feeds.

The present study aims to evaluate the potential probiotic effect of two beneficial bacterial strains towards *Artemia* by application of a challenge test with a virulent and with an opportunistic pathogenic *Vibrio* strain. In addition, by setting different gnotobiotic conditions (through a combination of yeast isogenic mutants and bacteria as feed), it is possible to perform these challenges with *Artemia* in different nutritional status.

2. Material and methods

2.1. Axenic cultures of yeast

Two strains of baker's yeast (*Saccharomyces cerevisiae*) were used as feed for *Artemia*: the wild type strain (WT) (BY4741, genotype *Mat a*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) and its *mn9* isogenic mutant (BY4741; genotype *Mat a*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; YPL050c::kanMX4). The *mn9* yeast was previously shown to be a better quality feed to *Artemia* than the WT yeast (Marques et al., 2004b). This isogenic mutant presents a null mutation resulting in a lower concentration of mannose, linked to mannoproteins, and

higher concentrations of chitin and glucans in the yeast cell wall (Magnelli et al., 2002; Marques et al., 2004b). Both strains were provided by the European *S. cerevisiae* Archive for Functional Analysis (EURO-SCARF, University of Frankfurt, Germany). Both yeast strains (WT and *mn9* YEPD) were cultured in a complete Yeast Extract Peptone Dextrose medium (YEPD); in addition the *mn9* yeast was cultured in a minimal Yeast Nitrogen Based medium (YNB) (*mn9* YNB). The procedures used in the present study to culture both yeasts were identical to the methods described by Marques et al. (2004b). Both strains cultured in YEPD were harvested by centrifugation ($\pm 800 \times g$ for 10 min) in the stationary growth phase (using a spectrophotometer to measure the optical density—OD—of the yeast culture at a wavelength of 600 nm after 3 days of culture; OD₆₀₀: ± 10.600 for WT and OD₆₀₀: ± 7.300 for *mn9*), while *mn9* cultured in YNB was harvested in the exponential growth phase (after 20 h of culture; OD₆₀₀: ± 0.700). All handlings were performed in a laminar-flow hood to maintain sterility. These three feed sources were chosen according to their nutritional quality to *Artemia*: poor-quality feed (WT yeast—enabling low *Artemia* survival and low growth), medium-quality feed (*mn9* yeast cultured in YEPD—enabling intermediate values of *Artemia* survival and growth) and good-quality feed (*mn9* yeast cultured in YNB—enabling high *Artemia* survival and growth) (Marques et al., 2004a,b, 2005). Yeasts were resuspended in filtered and autoclaved seawater (FASW, 0.2 μm) and their densities were determined by measuring twice the cell concentration, using a Bürker haemocytometer. Suspensions were stored at 4°C and used to feed *Artemia* until the end of each experiment.

2.2. Bacterial strains and growth conditions

Two bacterial strains (strain LVS 2—*Bacillus* spp.; and strain LVS 3—*Aeromonas hydrophila*) were selected for their positive effect towards *Artemia* (Verschuere et al., 1999, 2000; Marques et al., 2005) and examined for their ability to protect nauplii cultured in different gnotobiotic environments against two different pathogens. Furthermore, two bacteria pathogenic towards *Artemia*, namely *Vibrio proteolyticus* strain CW8T2 (Verschuere et al., 1999, 2000) and *Vibrio campbellii* strain LMG21363 (Soto-Rodriguez et al., 2003; Gomez-Gil et al., 2004) were also used in this study. Pure cultures of the 4 bacterial strains were obtained from the Laboratory of Microbial Ecology and Technology (LabMET), Ghent University, and from the

Laboratory of Microbiology, Ghent University. The bacterial strains were stored at -80°C and grown overnight at 28°C on marine agar (MA), containing Difco™ marine broth 2216 (37.4 g/l, BD Biosciences) and agar bacteriological grade (20 g/l, ICN). For each bacterial strain a single colony was selected from the plate and incubated overnight at 28°C in Difco™ marine broth 2216 on a shaker (150 rpm). Bacteria were harvested by centrifugation (15 min; $\pm 2200\times g$), the supernatant was discarded and the pellet was resuspended in 20 ml FASW. Bacterial densities were determined by spectrophotometry ($\text{OD}_{550}=1.000$ corresponds to 1.2×10^9 cells/ml; according to the McFarland standard, BioMerieux, Marcy L'Etoile, France, and confirmed in our study for all bacterial strains). Bacterial suspensions were stored at 4°C until the end of each experiment.

2.3. *Artemia* gnotobiotic culture

Experiments were performed with *Artemia franciscana* cysts, originating from Great Salt Lake, Utah, USA (EG® Type, INVE Aquaculture NV, Belgium). Bacteria-free cysts and nauplii were obtained via decapsulation according to the procedure described by Sorgeloos et al. (1986). During decapsulation 0.22- μm -filtered aeration was provided. All manipulations were carried out under a laminar flow hood and all necessary tools were previously autoclaved at 120°C for 20 min. Decapsulated cysts were washed with FASW over a 50 μm sterile filter net and transferred to a sterile 50 ml screw cap Falcon tube (TRP®, γ -irradiated) containing 30 ml of FASW. The tube was capped and placed on a rotator at 4 cycles per min, exposed to constant incandescent light ($\pm 41 \mu\text{Em}^{-2}$) at 28°C for 18–20 h. Twenty axenic nauplii (Instar II) were picked and transferred to sterile 50 ml tubes containing 30 ml of FASW, together with the amount of feed scheduled for day 1. In treatments where bacteria were used (beneficial or pathogenic), the bacterial suspension was added at a density of approximately 5×10^6 cells/ml, determined by spectrophotometry using the McFarland standard. Each treatment consisted of four tubes (replicates). Tubes were placed on a rotator at 4 cycles per min, exposed to constant incandescent light ($\pm 41 \mu\text{Em}^{-2}$) at 28°C , being transferred to the laminar flow just once per day for feeding. The daily feeding schedule was adapted from Coutteau et al. (1990) and Marques et al. (2004b) and is intended to provide ad libitum ratios, but avoiding excessive feeding as to not affect the water quality in the test tubes.

2.4. Methods used to verify axenity

Axenity of feed, decapsulated cysts and *Artemia* cultures were checked at the end of each experiment using a combination of plating and live counting, following the procedures of Marques et al. (2004a,b, 2005). Absence of bacteria was monitored by transferring 100 μl of culture medium to Petri plates with marine agar 2216 ($n=2$). Plates were incubated for 5 days at 28°C . As for live counting, each sample was stained with tetrazolium salt MTT (-3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, 0.5% w/v) in a sterile recipient (1 part of MTT to 9 parts of sample) and incubated at 30°C for 30 min. Under a light microscope (1000 \times magnification), live bacterial detection was performed. The MTT blue stains all viable/living cells remaining in the culture (Sladowski et al., 1993), facilitating the detection of bacterial contaminations. Contaminated culture tubes were not considered for further analysis and the experiment was repeated.

2.5. Experimental design

This study comprises 4 experiments and their experimental design is schematized in Fig. 1. In experiment 1, probiotic bacteria (LVS 2, LVS 3 or a mixture of LVS 2 and LVS 3 added in equal proportions, called MIX) were provided daily to *Artemia* (without being fed with yeast) and subsequently challenged with the pathogenic bacteria *V. campbellii* (VC) or *V. proteolyticus* (VP) daily until day 5 or only at day 3. As control treatment, *Artemia* were maintained only with VC or VP (provided daily or only at day 3), or only with an LVS strain added daily. In experiments 2–4, *Artemia* were fed respectively with WT yeast, mnn9 YEPD yeast and mnn9 YNB yeast: in these experiments, *Artemia* were fed with the yeast (added at day 1 and day 2—experiments A; or daily until day 5—experiments B) together with the putative probiotic bacteria (LVS 2, LVS 3 or MIX) before challenge with a pathogen (VC or VP). As control treatments, *Artemia* were only supplied with the feed without any bacteria; or supplied with the feed and with VC or VP at day 3; or fed with the yeast and with an LVS strain at day 1. The experiments were repeated in order to evaluate the reproducibility. In addition, *Artemia* performance of the control treatments (in the experiments 2–4) was compared to results previously obtained by Marques et al. (2004a,b, 2005). If significant differences were detected, data were not considered for further analysis and the experiment was repeated. In all experiments, no additional improvements in *Artemia* performance were

	Day 1 start	Day 2	Day 3	Day 4	Day 5	Day 6 harvest
Exp 1	a) NB	→	→	→	→	→
	b) B	→ B	→ B	→ B	→ B	→
	c) B	→ B	→ B+P	→ B	→ B	→
	d) NB	→	→ P	→	→	→
	e) P	→ P	→ P	→ P	→ P	→
	f) B+P	→ B+P	→ B+P	→ B+P	→ B+P	→
Exp 2A–4A	g) Y	→ Y	→	→	→	→
	h) Y+B	→ Y	→	→	→	→
	i) Y	→ Y	→ P	→	→	→
	j) Y+B	→ Y	→ P	→	→	→
Exp 2B–4B	k) Y	→ Y	→ Y	→ Y	→ Y	→
	l) Y+B	→ Y	→ Y	→ Y	→ Y	→
	m) Y	→ Y	→ Y+P	→ Y	→ Y	→
	n) Y+B	→ Y	→ Y+P	→ Y	→ Y	→

Fig. 1. Experimental design for the 4 experiments (Exp). Legend: Exp 2A–4A—the feed was added only at day 1 and day 2; Exp 2B–4B—the feed was added daily from day 1 until day 5; a)–n) correspond to the treatments performed; NB—no bacteria; B—beneficial bacteria (LVS 2, LVS 3 or MIX); P—pathogen (*V. campbellii* or *V. proteolyticus*); Y—yeast provided as feed: WT (Exp 2), mnn9 YEPD (Exp 3) and mnn9 YNB (Exp 4).

observed when nauplii were supplied with MIX in comparison to the use of only one beneficial bacterial strain (results not shown).

2.6. Survival and growth of *Artemia*

The survival percentage was determined daily for each treatment. For this purpose, the number of live *Artemia* was registered, before feeding or before adding bacteria, by exposing each transparent Falcon tube to an incandescent light without opening the tube to avoid contamination. At the end of each experiment (day 6 after hatching), live *Artemia* were fixed with lugol solution to measure their individual length (IL), using a dissecting microscope equipped with a drawing mirror, a digital plan measure and the software *Artemia 1.0*[®] (courtesy Marnix Van Damme). As a criterion that combines both the effects of survival and IL, the total biomass production (TBP) was determined according to the following equation: TBP (millimeters per Falcon tube—mm/FT)=number of survivors × mean IL.

2.7. Statistics

The percentages of larval survival were arcsine transformed, while values of IL and TBP were

logarithmic or square root transformed to satisfy normal distribution and homoscedasticity requirements. Differences in survival, IL and TBP of *Artemia* cultured in different conditions were investigated with *t*-test, analysis of variances (ANOVA) and Tukey's multiple comparisons range. All statistical analyses were tested at 0.05 level of probability, using the software Statistica 5.5[®] (Statsoft, Inc).

3. Results

3.1. *Artemia* fed only with selected bacteria

In a first challenge test with VC or VP, the effect of the beneficial LVS strains as the only feed was studied. Results presented in Table 1 (experiment 1; see also Fig. 1) show that the addition of these strains allows some non-challenged nauplii to survive until the end of the experiment (treatments b), although with low TBP (due to both higher survival and IL). *Artemia* challenged at day 3 with VC died within 24h independently of the bacterial strain used to feed the nauplii (treatments c). Some *Artemia* challenged at day 1 with VC could survive until day 3 either in the absence or presence of bacterial feed (treatment e). In the VP challenge test, in the absence of bacterial feed, *Artemia* died considerably

Table 1

Experiment 1—survival (%), individual length (IL) (mm) and total biomass production (TBP) (mm per Falcon tube—FT) of *Artemia* supplied only with LVS 2 or LVS 3 from day 1 to day 5 (D1–5)

Treatments		Survival (%)				IL (mm)	TBP (mm/FT)
		Day 3	Day 4	Day 5	Day 6		
a)	No bacteria	67±12 ^{ab}	7±5 ^{cd}	0 ^b	0 ^c	—	0.00 ^c
b)	LVS2 D1–5	60±7 ^{ab}	43±20 ^{ab}	34±13 ^a	21±5 ^a	1.26±0.24 ^a	5.36±1.21 ^a
b)	LVS3 D1–5	64±9 ^{ab}	59±5 ^a	35±6 ^a	11±5 ^{ab}	1.60±0.17 ^a	3.60±1.53 ^{ab}
c)	LVS2 D1–5+VC D3	60±7 ^{ab}	1±3 ^{de}	0 ^b	0 ^c	—	0.00 ^c
c)	LVS3 D1–5+VC D3	56±9 ^b	3±3 ^{de}	0 ^b	0 ^c	—	0.00 ^c
d)	VC D3	66±13 ^a	0 ^c	0 ^b	0	—	0.00 ^c
e)	VC D1–5	9±8 ^{ef}	0 ^c	0 ^b	0 ^c	—	0.00
f)	LVS2 D1–5+VC D1–5	20±8	0	0	0	—	0.00
f)	LVS3 D1–5+VC D1–5	6±5 ^f	3±3 ^{de}	3±3 ^b	3±3 ^{bc}	2.02±0.64 ^a	1.01±1.17 ^{bc}
c)	LVS2 D1–5+VP D3	74±17 ^{ab}	10±7 ^{cd}	0 ^b	0 ^c	—	0.00 ^c
c)	LVS3 D1–5+VP D3	56±9 ^b	28±6 ^b	0 ^b	0 ^c	—	0.00 ^c
d)	VP D3	80±14 ^a	6±3 ^{cd}	0 ^b	0 ^c	—	0.00 ^c
e)	VP D1–5	45±7 ^b	0 ^c	0 ^b	0 ^c	—	0.00 ^c
f)	LVS2 D1–5+VP D1–5	31±6 ^{cd}	15±7 ^{bc}	0 ^b	0 ^c	—	0.00 ^c
f)	LVS3 D1–5+VP D1–5	49±12 ^{bc}	26±3 ^b	0 ^b	0 ^c	—	0.00 ^c

D3—added only at day 3, VC—*Vibrio campbellii*, VP—*Vibrio proteolyticus*. Data provided are the means with the standard deviation (mean±S.D.). Values in the same column showing the same superscript letter are not significantly different ($p_{\text{Tukey}} > 0.05$). The first column in the table refers to the type of treatment (see Fig. 1).

slower compared to the VC challenge test (treatment d). The pathogenic nature of VP was also obvious when this bacterium was provided on day 3 (treatments c),

resulting in significant lower survival in comparison to pathogen-free nauplii (treatments b) independently from the selected bacteria used.

Table 2

Experiment 2—survival (%), individual length (IL) (mm) and total biomass production (TBP) (mm per Falcon tube—FT) of *Artemia* fed with the poor-quality wild type yeast (WT) only until day 2 (Exp 2A) or from day 1 to day 5 (Exp 2B), inoculated with LVS 2 or LVS 3 at day 1 (D1)

Treatments		Survival (%)				IL (mm)	TBP (mm/FT)
		Day 3	Day 4	Day 5	Day 6		
<i>Exp 2A—feeding only until day 2</i>							
g)	WT	34±6 ^b	29±6 ^{cd}	20±6 ^{bc}	13±6 ^b	1.52±0.26 ^{ab}	3.80±1.96 ^{bc}
h)	WT+LVS2 D1	60±11 ^a	56±9 ^a	56±9 ^a	56±9 ^a	1.52±0.31 ^{ab}	17.10±2.60 ^a
h)	WT+LVS3 D1	58±10 ^a	41±9 ^{abc}	24±3 ^b	6±13 ^{bc}	1.18±0.16 ^b	1.48±2.95 ^{bcd}
i)	WT+VC D3	55±14 ^a	5±6 ^{ef}	0 ^e	0 ^c	—	0.00 ^d
j)	WT+LVS2 D1+VC D3	54±8 ^a	26±12 ^{cd}	10±9 ^{cd}	3±3 ^c	1.72±0.05 ^a	0.86±0.99 ^{cd}
j)	WT+LVS3 D1+VC D3	55±9 ^a	20±11 ^{de}	4±5 ^{de}	0 ^c	—	0.00 ^d
i)	WT+VP D3	54±9 ^a	1±3 ^f	0 ^c	0 ^c	—	−0.00 ^d
j)	WT+LVS2 D1+VP D3	61±8 ^a	51±13 ^{ab}	24±9 ^{bc}	11±3 ^b	1.79±0.23 ^a	4.03±0.90 ^b
j)	WT+LVS3 D1+VP D3	59±10 ^a	35±11 ^{bcd}	24±6 ^{bc}	8±3 ^b	1.69±0.44 ^{ab}	2.54±0.98 ^{bc}
<i>Exp 2B—feeding days 1–5</i>							
k)	WT	58±9 ^b	34±9 ^c	25±7 ^c	23±6 ^d	1.56±0.22 ^{ab}	7.02±2.01 ^c
l)	WT+LVS2 D1	76±6 ^a	75±7 ^a	75±7 ^a	68±14 ^{ab}	1.89±0.27 ^a	25.52±5.46 ^{ab}
l)	WT+LVS3 D1	56±8 ^b	55±6 ^b	55±6 ^b	49±3 ^c	2.01±0.37 ^a	19.60±1.01 ^b
m)	WT+VC D3	55±7 ^b	6±3 ^e	1±3 ^d	0 ^f	—	0.00 ^e
n)	WT+LVS2 D1+VC D3	48±13 ^b	25±11 ^{cd}	8±3 ^d	1±3 ^{ef}	1.41 ^b	0.35±0.71 ^{de}
n)	WT+LVS3 D1+VC D3	56±5 ^b	11±3 ^{de}	5±6 ^d	0 ^f	—	0.00 ^e
m)	WT+VP D3	54±5 ^b	31±3 ^c	26±8 ^c	16±13 ^{de}	1.57±0.38 ^{ab}	5.10±4.13 ^{cd}
n)	WT+LVS2 D1+VP D3	81±10 ^a	79±11 ^a	79±11 ^a	79±11 ^a	1.73±0.32 ^{ab}	27.25±3.84 ^a
n)	WT+LVS3 D1+VP D3	68±3 ^{ab}	66±3 ^a	65±4 ^{ab}	61±5 ^b	1.90±0.39 ^a	23.28±1.82 ^a

D3—added only at day 3, VC—*Vibrio campbellii*, VP—*Vibrio proteolyticus*. Data provided are the means with the standard deviation (mean±S.D.). Values in the same column showing the same superscript letters are not significantly different ($p_{\text{Tukey}} > 0.05$). The first column in the table refers to the type of treatment (see Fig. 1).

3.2. *Artemia* fed with poor-quality yeast

Performance of *Artemia* fed with the poor-quality WT yeast in the presence or absence of LVS 2, LVS 3 or MIX and challenged with VC or VP is presented in Table 2 (experiment 2; see also Fig. 1). *Artemia* supplied solely with WT yeast presented lower TBP when fed only until day 2 (treatment g) than nauplii fed daily (treatment k), mostly due to higher survival, although differences were not significant ($n=4$; $p=0.060$). Statistical improvements in TBP were observed in *Artemia* fed daily and inoculated with the selected bacteria (treatments l) in comparison to the bacteria-free control (treatment k), due both to higher survival and IL. Yet, when nauplii was fed until day 2 with WT yeast, only treatments provided with LVS 2 (treatment h) yielded significantly improved *Artemia* performance in comparison to the bacteria-

free control (treatment g), mostly due to higher survival. *Artemia* challenged with VC (Exp 2A and 2B) or VP (Exp 2A) and not supplied with selected bacteria died before day 5 (Fig. 2). Yet, some *Artemia* supplied daily with WT yeast and challenged with VP survived until day 6 (Exp 2B) revealed no significant differences in TBP in comparison to the pathogen bacteria-free control. The addition of selected bacteria in experiments 2A and 2B could hardly protect *Artemia* in a challenge test with VC (no survivors on day 6; treatments j and n) (Table 2). Only nauplii supplied with LVS 2 presented significantly higher survival at day 4. Yet, when selected bacteria were supplied to nauplii and challenged with VP (treatments j and n), significantly higher TBP were registered in comparison to challenged nauplii not supplied with the selected bacteria (treatments i and m), mostly due to higher survival. Under such

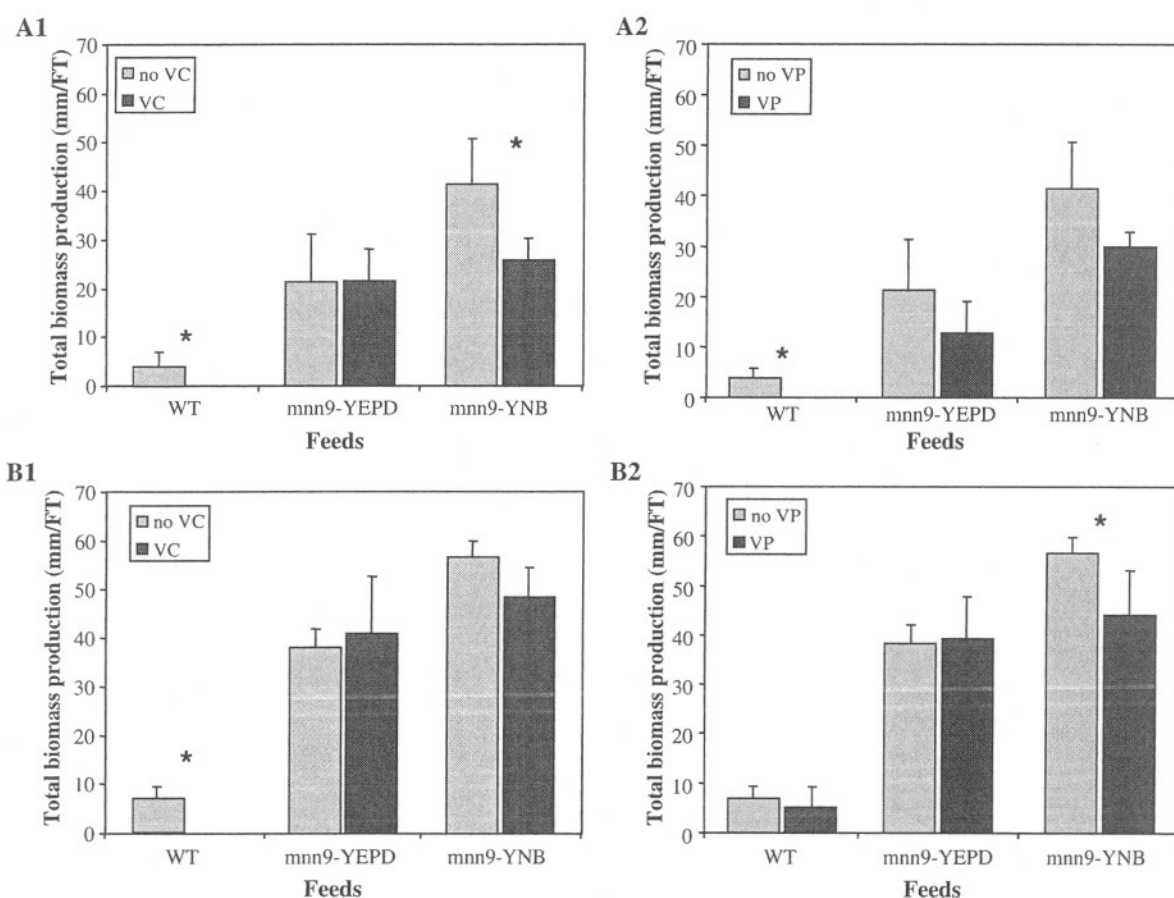


Fig. 2. Histogram of the average *Artemia* total biomass production (in mm/Falcon tube, and respective standard deviation) when fed with the 3 feeds only until day 2 (A1 and A2) or daily until day 5 (B1 and B2). Grey bars represent results of nauplii not supplied with the pathogen, while black bars represent nauplii challenged with pathogens at day 3. Graphs A1 and B1 corresponds to *V. campbellii* (VC), while graphs A2 and B2 refers to *V. proteolyticus* (VP). Asterisks mean significant differences between the two bars ($p_{t-test} < 0.05$).

conditions VP does not behave as a pathogen (compare treatment k and m).

3.3. *Artemia* fed with medium-quality yeast

According to Table 3 (experiment 3), *Artemia* supplied solely with the medium-quality mnn9 YEPD yeast presented significantly lower TBP when fed only until day 2 (treatment g) than nauplii fed daily (treatment k) ($n=4$; $p=0.018$), due equally to lower survival and IL. The addition of selected bacteria significantly improved TBP of *Artemia* fed mnn9 YEPD yeast (treatments h and l), due to both higher survival and IL. *Artemia* challenged with VC or VP and not supplied with the beneficial bacteria (treatments i and m) performed as good as unchallenged *Artemia* (treatments g and k) (Fig. 2). The addition of beneficial bacteria to challenged *Artemia* fed daily with this yeast (Exp 3B; treatments n) did not enhance significantly their performance. Yet, challenged *Artemia* cultured under starvation conditions (Exp 3A—especially with VP) and inoculated with selected bacteria (treatments j) presented higher TBP (although not always statistically significant) in comparison to

treatments where only pathogens were provided (treatments i) (Table 3).

3.4. *Artemia* fed with good-quality yeast

Artemia supplied solely with the good-quality mnn9 YNB yeast (Table 4—experiment 4) presented significantly lower TBP when fed only until day 2 (treatment g) in comparison to nauplii fed daily (treatment k) ($n=4$, $p=0.022$). In the presence of selected bacteria (treatments h and l), *Artemia* performance presented no significant improvements in comparison to the bacteria-free control (treatments g and k). The addition of LVS 3 (Exp 4A) significantly reduced TBP, mostly due to lower IL. When fed with this yeast, *Artemia* challenged with VC or VP and not supplied with selected bacteria (treatments i and m) revealed generally lower TBP in comparison to the pathogen-free control (treatments g and k) (Fig. 2). Yet, significant differences were only observed in Exp 4A when challenged with VC, mostly due to lower survival. Generally, the inoculation of selected bacteria to challenged nauplii (treatments j and n) could not significantly alter their TBP in comparison to treatments where only pathogens were provided

Table 3

Experiment 3 — survival (%), individual length (IL) (mm) and total biomass production (TBP) (mm per Falcon tube — FT) of *Artemia* fed with the medium-quality mnn9 yeast cultured in YEPD only until day 2 (Exp 3A) or from day 1 to day 5 (Exp 3B), inoculated with LVS 2 or LVS 3 at day 1 (D1)

Treatments		Survival (%)				IL (mm)	TBP (mm/FT)
		Day 3	Day 4	Day 5	Day 6		
<i>Exp 3A — feeding only until day 2</i>							
g)	mnn9 YEPD	78±9 ^a	55±15 ^b	48±21 ^{bcd}	45±21 ^{bcd}	2.36±0.42 ^a	21.24±10.01 ^{de}
h)	mnn9 YEPD+LVS2 D1	86±3 ^a	71±9 ^{ab}	70±7 ^{ab}	68±6 ^{ab}	2.88±0.46 ^a	38.88±3.72 ^{ab}
h)	mnn9 YEPD+LVS3 D1	84±13 ^a	80±7 ^a	80±7 ^a	75±7 ^a	3.00±0.57 ^a	45.00±4.24 ^a
i)	mnn9 YEPD+VC D3	88±13 ^a	79±15 ^{ab}	55±18 ^{bcd}	46±14 ^{cd}	2.32±0.32 ^a	21.46±6.66 ^{de}
j)	mnn9 YEPD+LVS2 D1+VC D3	88±5 ^a	76±12 ^{ab}	59±9 ^{bc}	54±10 ^{bc}	2.58±0.60 ^a	27.74±5.32 ^{cd}
j)	mnn9 YEPD+LVS3 D1+VC D3	84±13 ^a	66±13 ^{ab}	56±5 ^c	56±5 ^{bc}	3.21±0.55 ^a	36.11±3.07 ^{bc}
i)	mnn9 YEPD+VP D3	85±4 ^a	70±10 ^{ab}	30±12 ^d	26±13 ^d	2.41±0.39 ^a	12.65±6.34 ^e
j)	mnn9 YEPD+LVS2 D1+VP D3	85±11 ^a	70±11 ^{ab}	51±12 ^{cd}	49±14 ^{bcd}	3.03±0.47 ^a	29.54±8.70 ^{bcd}
j)	mnn9 YEPD+LVS3 D1+VP D3	88±12 ^a	69±22 ^{ab}	68±23 ^{abc}	66±25 ^{abc}	3.28±0.79 ^a	43.46±16.15 ^{abcd}
<i>Exp 3B — feeding from days 1–5</i>							
k)	mnn9 YEPD	86±3 ^{ab}	86±3 ^{ab}	78±9 ^{bc}	75±7 ^{bc}	2.55±0.35 ^b	38.25±3.61 ^d
l)	mnn9 YEPD+LVS2 D1	95±4 ^a	95±4 ^a	93±3 ^a	93±3 ^a	3.70±0.66 ^a	68.45±2.14 ^a
l)	mnn9 YEPD+LVS3 D1	84±9 ^{ab}	84±9 ^{ab}	84±9 ^{abc}	84±9 ^{abc}	3.22±0.74 ^{ab}	53.94±6.10 ^c
m)	mnn9 YEPD+VC D3	84±13 ^{ab}	79±18 ^{ab}	70±20 ^{abc}	70±20 ^{abc}	2.93±0.69 ^{ab}	41.02±11.72 ^{cd}
n)	mnn9 YEPD+LVS2 D1+VC D3	88±8 ^{ab}	88±8 ^{ab}	88±8 ^{ab}	88±8 ^{ab}	3.84±0.72 ^a	67.84±5.87 ^{ab}
n)	mnn9 YEPD+LVS3 D1+VC D3	74±17 ^{ab}	74±17 ^{ab}	61±24 ^{bc}	61±24 ^{bc}	3.16±0.69 ^{ab}	38.71±15.13 ^{cd}
m)	mnn9 YEPD+VP D3	76±14 ^b	71±16 ^b	65±11 ^c	63±13 ^c	3.15±0.58 ^{ab}	39.38±8.33 ^d
n)	mnn9 YEPD+LVS2 D1+VP D3	85±11 ^{ab}	78±6 ^b	73±13 ^{bc}	73±13 ^{bc}	3.67±0.52 ^a	53.22±9.24 ^{bc}
n)	mnn9 YEPD+LVS3 D1+VP D3	90±4 ^{ab}	85±4 ^{ab}	84±5 ^{ab}	74±17 ^{abc}	2.92±0.63 ^{ab}	43.07±9.94 ^{cd}

D3—added only at day 3, VC—*Vibrio campbellii*, VP—*Vibrio proteolyticus*. Data provided are the means with the standard deviation (mean±S.D.). Values in the same column showing the same superscript letters are not significantly different ($p_{\text{Tukey}} > 0.05$). The first column in the table refers to the type of treatment (see Fig. 1).

Table 4

Experiment 4—Daily survival (%), individual length (IL) (mm) and total biomass production (TBP) (mm per Falcon tube—FT) of *Artemia* fed with the good-quality mnn9 yeast cultured in YNB only until day 2 (Exp 4A) or from day 1 to day 5 (Exp 4B), inoculated with LVS 2 or LVS 3 at day 1 (D1)

Treatments		Survival (%)				IL (mm)	TBP (mm/FT)
		Day 3	Day 4	Day 5	Day 6		
<i>Exp 4A—Feeding only until day 2</i>							
g)	mnn9 YNB	89±10 ^a	89±10 ^a	85±15 ^{ab}	83±18 ^{ab}	2.51±0.43 ^a	41.42±9.28
h)	mnn9 YNB+LVS2 D1	85±12 ^a	85±12 ^{ab}	85±12 ^a	85±12	2.98±0.47 ^a	50.66±6.88 ^a
h)	mnn9 YNB+LVS3 D1	71±8 ^a	45±4 ^d	43±6 ^d	40±4 ^d	2.83±0.54 ^a	22.64±2.31 ^d
i)	mnn9 YNB+VC D3	88±9 ^a	79±6 ^{ab}	73±10 ^{abc}	55±9 ^c	2.34±0.36 ^a	25.74±4.27 ^{cd}
j)	mnn9 YNB+LVS2 D1+VC D3	88±12 ^a	85±12 ^{ab}	83±12 ^{ab}	63±19 ^{abc}	2.70±0.50 ^a	33.75±10.46 ^{abcd}
j)	mnn9 YNB+LVS3 D1+VC D3	71±8 ^a	64±5 ^c	60±4 ^c	49±8 ^{cd}	2.48±0.49 ^a	24.18±3.72 ^{cd}
i)	mnn9 YNB+VP D3	83±9 ^a	78±10 ^{abc}	68±3 ^{bc}	61±6 ^{bc}	2.44±0.41 ^a	29.89±3.07 ^{bc}
j)	mnn9 YNB+LVS2 D1+VP D3	79±10 ^a	79±10 ^{abc}	73±9 ^{abc}	73±9 ^{ab}	2.92±0.65 ^a	42.34±5.06 ^a
j)	mnn9 YNB+LVS3 D1+VP D3	71±8 ^a	58±19 ^{bcd}	56±18 ^{abcd}	55±16 ^{bcd}	2.40±0.64 ^a	26.40±7.59 ^{bcd}
<i>Exp 4B—Feeding from Day 1–5</i>							
k)	mnn9 YNB	84±5 ^a	81±5 ^{ab}	81±5 ^a	81±5 ^a	3.48±0.71 ^a	56.55±3.33 ^{ab}
l)	mnn9 YNB+LVS2 D1	88±5 ^a	88±5 ^a	85±8 ^a	85±8 ^a	3.53±0.73 ^a	60.01±5.76 ^a
l)	mnn9 YNB+LVS3 D1	78±6 ^a	75±7 ^b	75±7 ^a	74±8 ^a	3.63±0.63 ^a	53.54±5.45 ^{abc}
m)	mnn9 YNB+VC D3	89±17 ^a	89±17 ^{ab}	75±10 ^a	74±9 ^a	3.28±0.49 ^a	48.38±6.21 ^{abc}
n)	mnn9 YNB+LVS2 D1+VC D3	78±12 ^a	74±11 ^{ab}	70±12 ^a	68±15 ^a	3.06±0.49 ^a	41.31±9.18 ^c
n)	mnn9 YNB+LVS3 D1+VC D3	84±14 ^a	81±15 ^{ab}	75±8 ^a	75±8 ^a	3.37±0.53 ^a	50.55±5.50 ^{abc}
m)	mnn9 YNB+VP D3	74±10 ^a	69±12 ^b	68±13 ^a	65±14 ^a	3.39±0.66 ^a	44.07±9.18 ^{bc}
n)	mnn9 YNB+LVS2 D1+VP D3	85±10 ^a	79±13 ^{ab}	79±13 ^a	79±13 ^a	3.39±0.73 ^a	53.39±8.92 ^{abc}
n)	mnn9 YNB+LVS3 D1+VP D3	85±11 ^a	84±9 ^{ab}	84±9 ^a	80±11 ^a	3.46±0.72 ^a	55.36±7.47 ^{abc}

D3—added only at day 3, VC—*Vibrio campbellii*, VP—*Vibrio proteolyticus*. Data provided are the means with the standard deviation (mean±S.D.). Values in the same column showing the same superscript letters are not significantly different ($p_{\text{Tukey}} > 0.05$). The first column in the table refers to the type of treatment (see Fig. 1).

(treatments i and m) (Table 4). Yet, TBP of nauplii fed until day 2 with mnn9 YNB, inoculated with LVS 2 and challenged with VP was significantly higher, due to both higher survival and IL.

4. Discussion

Generally, *Artemia* fed solely with bacteria (Table 1; treatments c–f) were able to resist longer to the presence of VP in comparison to VC, although with both pathogens the animals could not survive until day 6. In addition, *Artemia* fed daily with the poor-quality WT yeast was able to cope with VP, but not with VC (Table 2—treatment m). Similar results were obtained by Verschuere et al. (1999, 2000) for VP and by Marques et al. (2005) for both pathogens. These results clearly demonstrate the difference in pathogenicity of both vibrios to *Artemia* allowing to stipulate VP and VC as, respectively, an opportunistic and a virulent pathogen for the brine shrimp nauplii (Marques et al., 2005). It was interesting to note that *Artemia* fed daily with the virulent VC or with the opportunistic VP could survive until day 3 (Table 1; treatments e and f), while nauplii exposed to the same pathogens at day 3 died faster, especially with VC (treatments c and d). This effect can

probably be seen only under gnotobiotic conditions. The apparent higher resistance of *Artemia* in early developmental stages against pathogenic bacteria could be related to a variety of factors. One can speculate that probably fewer attaching sites for *Vibrio* are available in the naupliar gastrointestinal tract, not allowing those pathogens to adhere. Alternatively, the higher resistance in early developmental stages could be due to the presence of certain compounds sustaining naupliar resistance just after hatching. Stabili et al. (1999) detected lysozyme and two protease (trypsin-like) activities in *A. franciscana* cysts and envelope before hatching. Lysozyme is a well-known antibacterial substance (e.g. direct bacteriolytic action, phagocytosis stimulation) in organisms not able to produce immunoglobulins (Zachary and Hoffmann, 1984). Proteases are involved in protein digestion and in various aspects of immunology (e.g. killing phagocytosed pathogens) (Canicatti and D'Ancona, 1990). Therefore, lysozyme and proteases may influence the resistance of *Artemia* in early life stages against pathogens.

Providing *Artemia* with the bacteria (LVS 2 or LVS 3) generally counteracted the opportunistic VP (but not the virulent VC) when nauplii were supplied with feeds not able to provide total protection, such as: WT yeast

fed only until day 2 (Table 2; treatments i and j). These bacteria were even able to significantly improve *Artemia* performance when fed daily with the poor-quality WT yeast and challenged with VP, certainly by improving the animal's feeding condition (treatments m and n). No additional protection against VP or VC was observed when nauplii were provided with MIX in comparison to the use of only one beneficial LVS strain. In previous studies, Verschuere et al. (2000) reported prevention by LVS 2 and LVS 3 against VP, although *Artemia* were cultured in slightly different conditions: short-term experiments (48 h), pathogen inoculated at 10^3 cfu/ml and γ -irradiated feed were used. We could recently demonstrate (Marques et al., 2004b) that the quality of feed provided to *Artemia* is affected by the method used to sterilize the feed, such as γ -irradiation. *Artemia* cultured under starvation conditions (or with poor-quality feeds) generate weak animals that are vulnerable to diseases caused by opportunistic and pathogenic bacteria. The addition of probiotic bacteria can eventually cover the animal's needs (nutritionally or others) and, in this way, overcome the effects of opportunistic and pathogenic bacteria. Presumably, negative effects of opportunistic bacteria are easier to surmount when animals are cultured in better conditions (e.g. providing better quality feeds or beneficial bacteria) than the effects of real pathogens, due to different mechanisms used by the two tested pathogens. In this way, it seems more difficult to find probiotic strains to use against VC than against VP.

Generally, the daily addition of medium- or good-quality yeast to *Artemia*, without providing any selected bacteria, completely prevented detrimental effects from the opportunistic pathogen VP, and from the virulent pathogen VC (Fig. 2). Previous studies performed by Marques et al. (2005) reported similar protection of *Artemia* when fed with mnn9 yeast in comparison to WT yeast. Independently from the pathogenic bacteria used, the mnn9 yeast could act as a biological control agent of infections. This protection could be related to a better animal condition (and thus improved *Artemia* performance), caused by better nutritional quality of mnn9-based feeds in comparison to the WT yeast, or to a stimulation of the innate immune system. Yeast cells are surrounded by a cell wall composed mostly of β 1,3- and β 1,6-glucans, mannoproteins and chitin (Magnelli et al., 2002). The mannoproteins are mainly present in the outer cell wall layer (in WT yeast 48% of the cell wall is mannoproteins, according to Marques et al., 2004b). Since *Artemia* do not have digestive enzymes in their gut able to process these mannoproteins (Coutteau et al., 1990), the yeast content is probably

less accessible to other digestive enzymes, and thus the WT yeast cannot be properly digested. Consequently, animals fed with WT yeast present poor growth and survival, as was demonstrated by Marques et al. (2004a, b). The mnn9 mutation changes considerably the yeast cell wall composition, resulting in increased proportional presence of cell wall bound chitin and glucans in combination with reduced mannoproteins (Magnelli et al., 2002; Marques et al., 2004a,b). The distribution of the cell wall constituents also change (e.g. chitin is more evenly distributed through the cell wall in mnn9), causing an effect on the way the cell wall compounds are covalently linked to each other (Klis et al., 2002). This change increases cell wall permeability and decreased integrity (De Nobel et al., 1990), probably improving yeast digestibility to *Artemia*, thus allowing nauplii to perform better in comparison to WT yeast (Marques et al., 2004a,b). The better protection by mnn9 yeast might also be related to a stimulation of the innate immune system. In fact, some components presented in the yeast cell wall (β -glucans and chitin, offered in higher amounts to nauplii by feeding with mnn9) are likely to boost the non-specific immune response in *Artemia* against pathogenic bacteria, e.g. chitin (Sakai et al., 1992; Anderson and Siwicki, 1994) and β -glucans (Cerenius et al., 1994; Misra et al., 2004), conferring, in this way, protection to nauplii. Looking at the data across tables, it is clear that under gnotobiotic conditions *Artemia* can be cultured to a certain performance level (in terms of survival and/or growth) by offering different types of feed. When cultured with WT yeast cells in the presence of LVS 2 (Table 2; Exp 2B; treatment l), 68% of *Artemia* survived after 6 days. A similar value (75%) was obtained by feeding mnn9 YEPD (Table 3; Exp 3B; treatment k) cells. Yet, when challenged with VC, the former *Artemia* died (Table 2; Exp 2B; treatment n), while the latter did survive (Table 3; Exp 3B; treatment m). Although the survival/growth performance in these two gnotobiotic environments was very similar, only when fed mnn9 YEPD cells, *Artemia* is being conditioned to cope with a challenge of VC. The most plausible hypothesis that can be taken from these data is that the protective effect is caused by a change in the cell wall constituents (amount and/or cross-linking) and in particular β -glucans in mnn9 cells, rather than by a change in digestibility as similar survival/growth performance is noticed in the absence of a challenge. This hypothesis can only be verified by looking at differential expression of genes involved in the innate immune response (such as antimicrobial compounds, lysozyme and transglutaminase).

In the present study, *Artemia* fed with the good-quality yeast (mnn9 YNB) performed always better than animals fed with the medium-quality yeast (mnn9 YEPD), confirming findings of Marques et al. (2004b). Yet, the protection was not necessarily complete in animals fed with mnn9 YNB in comparison to animals fed with mnn9 YEPD (Fig. 2). The mnn9 YNB cells contain even higher amounts of glucans and chitin and decreased amounts of mannoproteins in the cell wall as compared to the cells of mnn9 YEPD yeast (Marques et al., 2004b). This result seems to indicate that the feeding condition of *Artemia* is not always linked with an improved protection against pathogenic bacteria. In line with the hypothesis that β -glucans are responsible for the protective effect through immunostimulation, an eventual overdose of immunostimulants (e.g. glucans) might have occurred, thus inducing immunosuppression, similarly as described in fish (Sakai, 1999).

In the case of a significant protection through the feed (mnn9 yeast), only in some cases additional probiotic effects were noticed: e.g. LVS 2 (VP: Exp 3A and 4A, treatment j; VC: Exp 3B, treatment n) or LVS 3 (VP and VC: Exp 3A, treatment j). In general, *Artemia* cultured in sub-optimal conditions with medium- and good-quality mnn9 yeast were less protected against both pathogens (Tables 3 and 4; treatment i) than nauplii fed daily ad libitum (treatments m), probably due to poorer animal conditions caused by the lack of feed. In such sub-optimal conditions, the simple addition of extra amounts of nutrients provided by the selected bacteria can explain the improvements in *Artemia* performance (Intriago and Jones, 1993; Marques et al., 2005), although this is less probable to have occurred with nauplii fed ad libitum with these feeds.

6. Conclusion

In conclusion, protection of nauplii against opportunistic and pathogenic bacteria is significantly improved by the quality of feed provided to *Artemia*. Only under poor feeding conditions, as with WT yeast cells, the probiotic effect of a bacterial strain against a weak pathogen such as VP can be demonstrated. Data suggest that the cell wall composition of mnn9 cells condition *Artemia*, allowing them to survive a VC challenge. The present experimental set-up allows to evaluate the true probiotic nature of a microorganism against virulent pathogens, under strict controlled conditions, as well as to screen virulence of different strains of pathogens. More studies are being performed to clarify the exact contribution of mnn9 yeast to the protection of *Artemia*

against VC and VP, using host gene-expression analysis, as these feeds can help to develop more successful and stable preventive solutions against disease outbreaks in aquaculture systems.

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